Gene Expression Profiling of the Leading Edge of Cutaneous Squamous Cell Carcinoma: IL-24-Driven MMP-7

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The precise mechanisms governing invasion at the leading edge of squamous cell carcinoma (SCC) and its subsequent metastasis are not fully understood. We aimed to define the cancer-related molecular changes that distinguish noninvasive tumor from invasive SCC. To this end, we combined laser capture microdissection with complementary DNA (cDNA) microarray analysis. We defined invasion-associated genes as those differentially regulated only in invasive SCC nests, but not in actinic keratosis or *in situ* SCC, compared with normal epidermis. There were 383 upregulated and 354 downregulated genes in the "invasion set." SCC invasion was characterized by aberrant expression of various proteolytic molecules. We noted increased expression of *MMP7* and *IL-24* in invasive SCC. IL-24 induced the expression of matrix metallopeptidase 7 (MMP7) in SCC cells in culture. In addition, blocking of MMP7 by a specific antibody significantly delayed the migration of SCC cells in culture. These results suggest a possible contribution of IL-24 to SCC invasion via enhancing focal expression of MMP7, although IL-24 has been suggested to have antitumor growth effects in other cancer types. Identification of regional molecular changes that regulate cancer invasion may facilitate the development of new targeted treatments for aggressive cancer.

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INTRODUCTION

Cutaneous squamous cell carcinoma (SCC), the second most frequent skin cancer, arises from interfollicular epidermal keratinocytes. Transformed malignant cells can proliferate in the epidermis as *in situ* SCC, eventually cross the basement membrane, and enter the dermis to form invasive SCC. Invasion to the dermis is a critical event, as cancer cells are allowed to access lymphatic and to a lesser degree blood vessels, which may result in metastasis. The American Joint Committee on Cancer, in fact, added tumor depth (>2-mm thickness or Clark level ⩾IV) as a high-risk feature of SCC (Farasat *et al.*, 2011). Although usually curable by resection, SCC accounts for the majority of ~10,000 deaths from nonmelanoma skin cancer in the United States each year (Weinberg *et al.*, 2007).

Cancer invasion can be mediated by several processes including degradation of extracellular matrix and growth and migration of tumor cells into surrounding stromal regions. Thus, studying the expression and regulation of tumor growth factors and proteolytic molecules within a tissue is important. Gene expression analysis, such as complementary DNA (cDNA) microarray analysis, has been applied to many cancer types including cutaneous SCC (Dooley *et al.*, 2003; Haider *et al.*, 2006; Kathpalia *et al.*, 2006; Nindl *et al.*, 2006; Hudson *et al.*, 2010; Padilla *et al.*, 2010). These prior studies identified numerous genes that might be involved in SCC pathogenesis. For example, we reported that matrix metallopeptidases (MMPs) including *MMP1*, *MMP10*, and *MMP13* were selectively expressed in SCC but not in psoriasis, a benign inflammatory skin disease characterized by epidermal hyperproliferation, but without invasion into the dermis by keratinocytes (Haider *et al.*, 2006). This suggests the potential importance of these MMPs in SCC progression.

Cytokines also have significant functions in tumor biology. T helper type 1 cytokines, such as IFN-γ, have been thought to possess antitumor properties, whereas T helper type 2 cytokines, such as IL-4, have primarily protumor activity. Recent

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Abbreviations: Ab, antibody; AK, actinic keratosis; cDNA, complementary DNA; FBS, fetal bovine serum; LCM, laser capture microdissection; MMP, matrix metallopeptidase; RT–PCR, reverse transcriptase–PCR; SCC, squamous cell carcinoma

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studies, however, reveal that the cytokines, such as IFN-γ, possess both anti- and pro-tumor activities depending on the tumor types as well as the tumor microenvironment (Zaidi and Merlino, 2011). IL-24, also known as melanoma differentiation associated gene-7 (mda-7), is a member of the IL-10 family of cytokines. It was initially found as a growth inhibitor of melanoma (Jiang *et al.*, 1995). Mounting evidence established a role of IL-24 in inducing apoptosis and cell death in many solid cancers including melanoma as well as epithelial carcinomas of various organs (Dash *et al.*, 2010). However, phase I clinical study using an adenovirus containing IL-24/mda-7 construct (Ad.IL24/mda-7, INGN241) was not able to control tumor growth of a penile, and a head and neck SCC (Cunningham *et al.*, 2005). IL-24 is known to be highly produced in psoriasis lesional skin (Rømer *et al.*, 2003). In addition, IL-24 was as potent as IL-22 to induce keratinocytic hyperplasia in a reconstituted human epidermis culture model (Sa *et al.*, 2007). It was thus speculated that IL-24 might have distinct functions in cutaneous SCC growth as opposed to antitumor functions established in other cancer types.

Cancer cells may change their phenotypes along with tumor progression, i.e., a switch of E-cadherin to N-cadherin expression (Hazan *et al.*, 2004) and an epithelial-to-mesenchymal transition in invading tumor cells (Thiery *et al.*, 2009). Cells at the invading front might, thus, be genomically distinct from bulk tumor cells. Therefore, we used laser capture microdissection (LCM) in order to collect subpopulations of SCC cells from human skin tissue. We generated the invasion signature gene set of cutaneous SCC, a set of genes that were differentially regulated in SCC invasive nests, but not in actinic keratosis (AK) or *in situ* SCC regions, compared with normal epidermis. We identified significant upregulation of *IL-24* and *MMP7* mRNA in the invading front of cutaneous SCC. Molecular interaction of these two molecules and their potential role in SCC progression are discussed in this study.

RESULTS

LCM combined with cDNA microarray analysis provides specific gene expression profiles for various stages of SCC progression

Tumor debulking samples were obtained during Mohs micrographic surgery for SCC. Three transformed epidermal regions in this study that represent the transition to invasive SCC were defined as follows: (1) AK (atrophic type), regions of severe dysplasia at the basal layer of atrophic epidermis with solar elastosis in dermis, (2) *in situ* SCC, tumor regions with transformed keratinocytes throughout the entire epidermis that have not crossed the basement membrane, and (3) invasive SCC, tumor nests that have invaded the dermis and disconnected from the bulk tumor mass (Figure 1a). There were 724 upregulated and 820 downregulated probe sets in AK, 1,042 upregulated and 1,200 downregulated probe sets in *in situ* SCC, and 1,325 upregulated and 1,461 downregulated probe sets in invasive SCC compared with microdissected normal epidermis (fold change >3.0 and false discovery rate <0.05; Figure 1a). A Venn diagram demonstrated 1,083 (503 upregulated and 580 downregulated) commonly regulated probe sets among the three regions, including *S100As*, *KRT6*, and *serpins* (Figure 1b and c). A group of genes that was selectively regulated in invasive SCC, but not in dysplasia or *in situ* SCC, was of particular interest as these genes might have significant roles in SCC invasion to the dermis. This consists of 383 upregulated and 354 downregulated probe sets and these genes were designated as invasion signature genes (Supplementary Table S1 online). The complete gene lists comparing each region with microdissected normal epidermis are found in Supplementary Tables S2–S4 online.

The invasion signature gene set characterized the tumor nests at the invasion front

Table 1 shows selected up- and down-regulated invasion signature genes. Genes encoding proteolytic molecules, such as *MMPs* and *PLAU*, were highly upregulated. A cell adhesion molecule *LAMC2* was also upregulated. The expression of *podoplanin* (*PDPN*) in cutaneous SCC was reported previously by quantitative reverse transcriptase–PCR (RT–PCR) and by immunohistochemistry (Schacht *et al.*, 2005; Moussai *et al.*, 2011). In contrast, melanocyte-related genes such as *TYRP1*, *DCT*, and *KIT*, as well as keratinocyte differentiation markers including *FLG*, *LOR*, and *LCE2B*, were downregulated. Ingenuity pathway analysis linked 28 canonical pathways to the SCC invasion signature gene set (Supplementary Table S5 online, Fisher's exact test, $P<0.05$). The most significant pathway was leukocyte extravasation signaling ($P=9.56\times10^{-4}$), followed by Aryl hydrocarbon receptor signaling ($P=1.46\times10^{-3}$), and hypoxia-inducible factor-1α signaling ($P=1.54\times10^{-3}$).

Regional expression of MMPs in the SCC tissue was mapped using preamplification quantitative RT–PCR

The expression of MMPs in SCC invasion nests was further investigated, as three of the top six genes in the invasion signature gene set were MMPs (*MMP3*, *MMP12*, and *MMP13*) and MMPs are critical for degrading the extracellular matrix surrounding tumor nests. Eleven MMPs were detected as differentially expressed probe sets along with SCC progression by microarray analysis (Table 2). The expression of *MMP9*, *MMP11*, and *MMP14* was increased even in AK. The expression of *MMP1*, *MMP2*, and *MMP10* started to elevate in *in situ* SCC, and further increased in invasive SCC by ~2- to 7-fold compared with *in situ* SCC. *MMP1* was the most abundant MMP in invasive SCC with a fold change = 107.82, followed by *MMP10* with a fold change = 48.35. The expression of *MMP3*, *MMP7*, *MMP12*, *MMP13*, and *MMP17* was selective for invasive SCC. The regional expression difference of all 23 known human *MMPs* was further tested using the same RNA used for microarray analysis by a more sensitive RT–PCR detection. A heat map clearly showed the increase of expression of multiple MMPs toward invasive SCC (Figure 2a). Out of 23 genes tested, 12 had significant difference among the four keratinocytic regions ($P<0.05$, Figure 2b–e and Supplementary Figure S1 online). Of these 12 genes, 11 were upregulated in cancer regions compared with normal epidermis or AK. The expression of *MMP28* was lower in *in situ* SCC than in normal epidermis. This was consistent with a previous

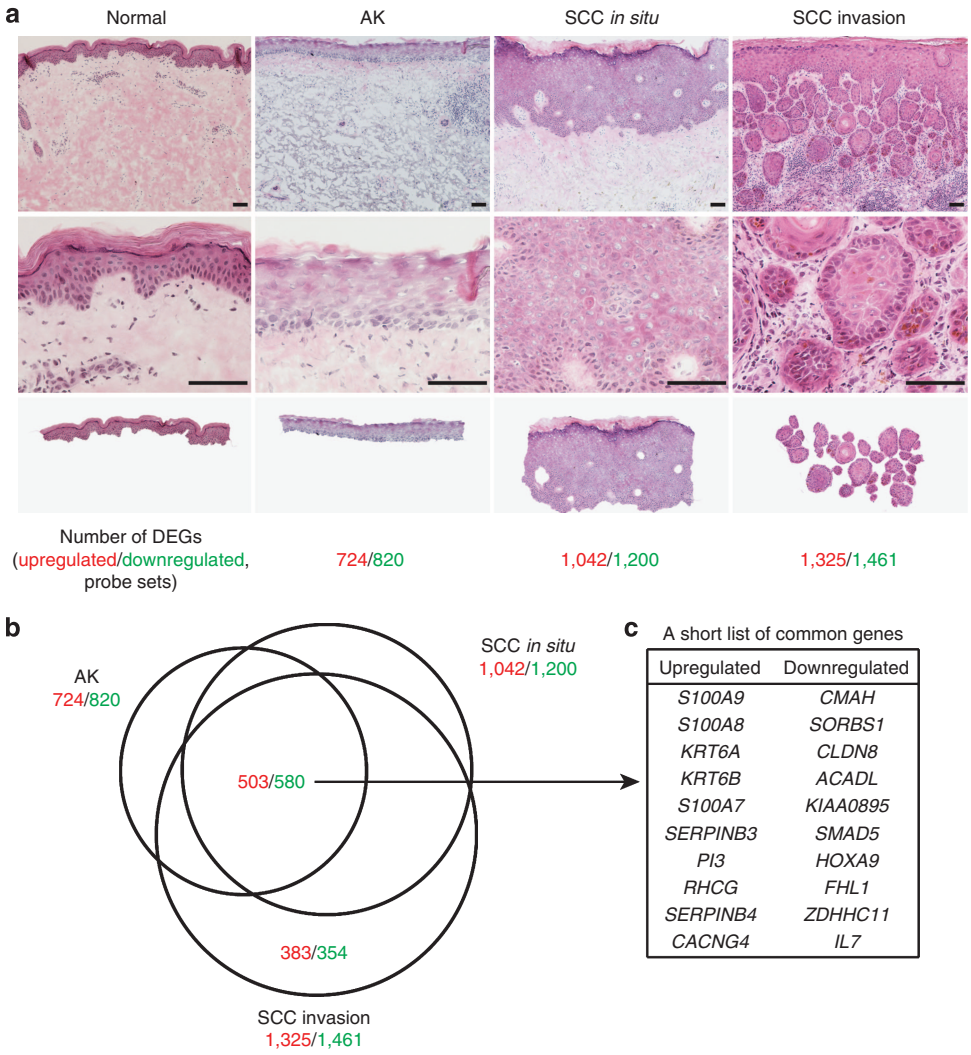

Figure 1. Combined laser capture microdissection (LCM) and complementary DNA (cDNA) microarray analysis identified region-specific gene expression changes in the squamous cell carcinoma (SCC) tissues. (**a**) Images of hematoxylin and eosin (H&E) staining for each region and number of differentially expressed probe sets identified in the corresponding regions compared with normal epidermis. Top panels indicate lower magnification, middle panels indicate higher magnifications, and bottom panels indicate images of LCM. Bar = 100 μm. AK, actinic keratosis. (**b**) A Venn diagram revealed the numbers of commonly regulated probe sets among the dysplasia/cancer regions compared with normal epidermis as well as uniquely regulated probe sets in the SCC invasion nests. (**c**) The top 10 common up- and downregulated probe sets among the three dysplasia/cancer regions compared with normal epidermis are listed. The numbers in red indicate upregulated probe sets, whereas those in green indicate downregulated probe sets. DEGs, differentially expressed genes.

report showing the specific expression of MMP28 in proliferating keratinocytes during wound healing, but not in SCC (Saarialho-Kere *et al.*, 2002). The mRNA of *MMP8, MMP20, MMP*23B, *MMP26, and MMP27* was rarely detected in any keratinocytic regions tested (Supplementary Figure S1 online). In addition, proportional odds model identified 14 MMPs as statistically significant (* in Figure 2a, *P*-values in Supplementary Table S6 online). This indicates that gene expression of these MMPs increases the odds of being in a phenotype of higher degree of malignancy. The results of cDNA microarray and preamplification RT–PCR were positively correlated as evidenced by Pearson's $r = 0.788$ ($P < 0.0001$), when the 11 genes detected in both methods were compared (Supplementary Figure S2 online).

Altered protein expression of the selected genes was confirmed by immunohistochemistry

Immunohistochemistry was performed in order to confirm the altered protein expression of the corresponding genes within tissue. MMP1, the most upregulated MMP on our gene list, strongly stained both SCC tumor nests and the interstitial region surrounding tumor nests (Figure 2f–g). Staining of MMP3 and MMP7 was selective in the invading cancer cells, but not in the epidermis of normal skin or the epidermis above the tumor nests (Figure 2h–k), thus confirming the specificity and gradual increase of the mRNA expression in tumor nests. MMP10 strongly stained tumor nests in the dermis, but it also stained the basal layer of keratinocytes in normal skin (Figure 2l–m). These genomic and protein data

Table 1. Selected (a) upregulated and (b) downregulated SCC invasion signature genes

	AK		SCC *in situ*		SCC invasion		
Symbol	**FCH**	**FDR**	**FCH**	**FDR**	**FCH**	**FDR**	**Description**
(a) Selected upregulated SCC invasion signature genes							
MMP12	1.14	0.90	2.31	0.37	**32.97**	**<0.01**	*Matrix metallopeptidase 12*
PTHLH	0.76	0.67	3.09	0.05	**17.89**	**<0.01**	*Parathyroid hormone-like hormone*
MMP3	1.19	0.89	4.77	0.15	**16.38**	**<0.01**	*Matrix metallopeptidase 3*
AIM2	1.80	0.34	2.72	0.08	**14.61**	**<0.01**	*Absent in melanoma 2*
NEFL	0.87	0.83	2.23	0.13	**13.34**	**<0.01**	*Neurofilament, light polypeptide*
MMP13	0.96	0.95	2.37	0.11	**12.53**	**<0.01**	*Matrix metallopeptidase 13*
KRT19	1.17	0.78	2.80	0.02	**11.33**	**<0.01**	*Keratin 19*
INHBA	0.86	0.80	2.42	0.07	**10.89**	**<0.01**	*Inhibin, βA*
IL7R	1.62	0.36	2.86	0.03	**9.45**	**<0.01**	*Interleukin 7 receptor*
CXCL10	3.63	0.24	5.26	0.11	**9.44**	**0.02**	*Chemokine (C-X-C motif) ligand 10*
SPP1	1.45	0.70	2.18	0.37	**8.76**	**<0.01**	*Secreted phosphoprotein 1*
PLAU	2.60	<0.01	2.73	<0.01	**8.45**	**<0.01**	*Plasminogen activator, urokinase*
PDPN	0.41	0.01	2.11	0.03	**8.04**	**<0.01**	*Podoplanin*
KRT18	2.24	0.16	2.80	0.06	**7.69**	**<0.01**	*Keratin 18*
FN1	0.67	0.61	3.30	0.09	**7.06**	**<0.01**	*Fibronectin 1*
APOBEC3G	2.45	0.03	2.55	0.02	**6.96**	**<0.01**	*Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G*
LAMC2	0.79	0.69	1.17	0.77	**6.77**	**<0.01**	*Laminin, γ2*
IL24	1.20	0.79	2.03	0.23	**6.74**	**<0.01**	*Interleukin 24*
CXCR4	2.60	0.09	1.78	0.31	**6.16**	**<0.01**	*Chemokine (C-X-C motif) receptor 4*
MMP7	1.09	0.89	1.16	0.77	**5.43**	**<0.01**	*Matrix metallopeptidase 7*
(b) Selected downregulated SCC invasion signature genes							
TYRP1	0.79	0.88	0.45	0.54	**0.03**	**<0.01**	*Tyrosinase-related protein 1*
FLG	0.60	0.70	0.14	0.09	**0.04**	**<0.01**	*Filaggrin*
LOR	1.39	0.80	0.25	0.21	**0.07**	**0.01**	*Loricrin*
TFAP2B	1.17	0.83	0.36	0.10	**0.07**	**<0.01**	*Transcription factor AP-2β*
HPGD	0.41	0.18	0.29	0.05	**0.08**	**<0.01**	*Hydroxyprostaglandin dehydrogenase*
DCT	1.24	0.81	0.31	0.11	**0.08**	**<0.01**	*Dopachrome tautomerase*
NFIB	0.61	0.38	0.36	0.04	**0.10**	**<0.01**	*Nuclear factor I/B*
CYorf15B	0.40	0.38	0.23	0.12	**0.11**	**0.02**	*Chromosome Y open reading frame 15B*
DDAH1	1.18	0.74	0.73	0.50	**0.12**	**<0.01**	*Dimethylarginine dimethylaminohydrolase 1*
CHI3L1	0.76	0.73	0.33	0.11	**0.12**	**<0.01**	*Chitinase 3-like 1*
LCE2B	1.15	0.87	0.25	0.05	**0.13**	**<0.01**	*Late cornified envelope 2B*
ANXA9	1.17	0.81	0.54	0.26	**0.13**	**<0.01**	*Annexin A9*
NEB	1.06	0.90	0.41	0.02	**0.13**	**<0.01**	*Nebulin*
ALOX12	1.26	0.47	0.38	<0.01	**0.14**	**<0.01**	*Arachidonate 12-lipoxygenase*
CEP76	0.46	0.09	0.40	0.04	**0.14**	**<0.01**	*Centrosomal protein 76 kDa*
ACSBG1	0.93	0.89	0.38	0.02	**0.15**	**<0.01**	*Acyl-CoA synthetase bubblegum family member 1*
KIT	0.64	0.43	0.42	0.09	**0.16**	**<0.01**	*v-kit Hardy-Zuckerman 4 feline Sarcoma viral oncogene homolog*
NFASC	0.38	<0.01	0.36	0.00	**0.16**	**<0.01**	*Neurofascin homolog*
AADAC	1.34	0.54	0.47	0.08	**0.16**	**<0.01**	*Arylacetamide deacetylase*
ATP6V0E2	0.80	0.55	0.38	0.00	**0.17**	**<0.01**	*ATPase, H+ transporting V0 subunit e2*

Abbreviations: AK, actinic keratosis; FCH, fold change; FDR, false discovery rate; SCC squamous cell carcinoma.
FCH and FDR in SCC invasion are in bold.

Table 2. MMP expression across regions measured by cDNA microarray analysis

	AK		SCC *in situ*		SCC invasion	
Symbol	FCH	FDR	FCH	FDR	FCH	FDR
MMP9	**3.42**	0.03	**3.85**	0.01	**19.31**	<0.01
MMP11	**3.05**	<0.01	**5.57**	<0.01	**5.77**	<0.01
MMP14	**3.08**	<0.01	**4.06**	<0.01	**3.91**	<0.01
MMP1	1.28	0.87	**16.61**	0.02	**107.82**	<0.01
MMP2	1.05	0.93	**5.85**	<0.01	**10.50**	<0.01
MMP10	1.14	0.89	**7.21**	<0.01	**48.35**	<0.01
MMP3	1.19	0.89	4.77	0.15	**16.38**	<0.01
MMP7	1.09	0.89	1.16	0.77	**5.43**	<0.01
MMP12	1.14	0.90	2.31	0.37	**32.97**	<0.01
MMP13	−1.04	0.95	2.37	0.11	**12.53**	<0.01
MMP17	2.62	<0.01	2.81	<0.01	**3.50**	<0.01

Abbreviations: AK, actinic keratosis; cDNA, complementary DNA; FCH, fold change; FDR, false discovery rate; MMP, matrix metallopeptidase; SCC squamous cell carcinoma.
FCH for the genes that satisfied the differential expression criteria is shown in bold.

provide the localization of MMPs within the cutaneous SCC tissues.

IL-24 was increased in SCC invasion front and upregulated the expression of MMP7 in SCC cells *in vitro*

IL-24 was upregulated in our SCC invasion signature gene set (Table 1a). The mRNA and protein expression of *IL-24* as well as its receptor subunits (*IL-20R1*, *IL-20R2*, and *IL-22R1*) within the tissues was confirmed. The expression of *IL-24* mRNA was detected only in *in situ* SCC and invasive SCC, but not in AK (Figure 3a). An IL-24 antibody detected signals in SCC tumor nests, but not normal skin (Figure 3b and c). The mRNA of the three receptor subunits was constitutively expressed in all regions, although *IL20R1* was slightly decreased in invasive SCC (Figure 3d–f). It has been reported that some cytokines, including transforming growth factor-α, enhance the expression of IL-24 in HaCaT cells as well as normal human epidermal keratinocytes (Poindexter *et al.*, 2010). The expression of *IL-24* mRNA in two human cutaneous SCC cell lines was thus investigated (see detailed descriptions of these cells in the Supplementary Materials and Methods online). SCC13 cells (Rheinwald and Beckett, 1981) constitutively expressed *IL-24* mRNA without addition of any cytokines. This expression was further increased by stimulation with transforming growth factor-α, tumor necrosis factor-α, and IFN-γ (Figure 3g). A431 cells, another SCC cell line (Giard *et al.*, 1973; Price *et al.*, 1988), did not express *IL-24* regardless of the stimuli (data not shown). IL-24 utilizes the same receptors to activate cells as IL-20 (Sabat, 2010), and IL-20 has been reported to upregulate the expression of *MMP7* in HaCaT cells (Wang *et al.*, 2006). Hence, we hypothesized that IL-24 might play a role in upregulating the expression of *MMP7* mRNA in SCC. *MMP7* mRNA in A431 or HaCaT cells was increased when cultured with 40 or 100 ng ml^{-1} of IL-24, or 100 ng ml^{-1} of IL-20 for 24 hours (Figure 3h and i). *MMP7* mRNA was not increased in SCC13 cells with the addition of IL-20 or IL-24 (Figure 3j), although SCC13 cells expressed *IL-24* mRNA constitutively. This might, in part, be explained by significantly lower expression of the IL-24 receptor subunits in SCC13 cells as compared with others (Supplementary Figure S3 online). Nevertheless, these data suggest a role of IL-24 in enhancing the expression of *MMP7* in SCC cells that may contribute to cancer progression.

Blocking of MMP7 by a specific antibody significantly delayed the migration of A431 cells

Finally, the function of MMP7 in A431 cells was examined using a scratch assay. A431 cells were selected as this cell line expresses ~50-fold higher *MMP7* mRNA than the other two cell lines tested (Figure 3h–j). A scratch was made when A431 cells reached 90% confluence, and they were maintained in the 0.1% fetal bovine serum (FBS)–containing media with or without an MMP7 antibody (MMP7Ab). The MMP7Ab used in this experiment was shown to block the activity of MMP7 (Ito *et al.*, 2007). Figure 4a–d shows the gap between cells after a 36-hour treatment with phosphate-buffered saline alone (Figure 4a) or different concentrations of the MMP7Ab (20 ng ml^{-1} (Figure 4b), 200 ng ml^{-1} (Figure 4c), and 2,000 ng ml^{-1} (Figure 4d)). The effect of blocking MMP7 was evaluated by calculating a percent confluence for each condition at each time point. The MMP7Ab significantly delayed the migration of A431 cells at a concentration of 2,000 ng ml^{-1} as compared with the other conditions (Figure 4e). This was also true after a 24-hour treatment with the MMP7Ab treatment at 2,000 ng ml^{-1} compared with phosphate-buffered saline and 20 ng ml^{-1} MMP7Ab (Figure 4f). A similar effect was observed when the A431 cells were maintained in media containing 10% FBS (Supplementary Figure S4 online). These results suggest the involvement of MMP7 in the migration of cutaneous SCC.

DISCUSSION

Previous studies including our own have examined gene expression profiling of cutaneous SCC using total RNA extracted from full-thickness tumor tissues (Dooley *et al.*, 2003; Haider *et al.*, 2006; Kathpalia *et al.*, 2006; Nindl *et al.*, 2006; Hudson *et al.*, 2010; Padilla *et al.*, 2010). To our knowledge, no studies combining LCM and cDNA microarray analysis on cutaneous SCC have been published. This study confirmed many genes of previous findings, i.e., the enhanced expression of epidermal differentiation complex genes, such as *S100As*, *SPRRs*, *IVL*, and *LCE3D*. Hudson *et al.* (2010) proposed the expression of *KRT13* as a marker of SCC. In this study, we found that *KRT13* is specific to *in situ* and invasive SCCs, but not AK. Thus, our study is unique in localizing hundreds of gene products to different stages of tumor progression *in vivo* by combining LCM and cDNA microarray analysis. This helps to identify molecular interactions that may happen in focal regions of SCC. One such example is the focal regulation of MMP7 by IL-24 cytokine.

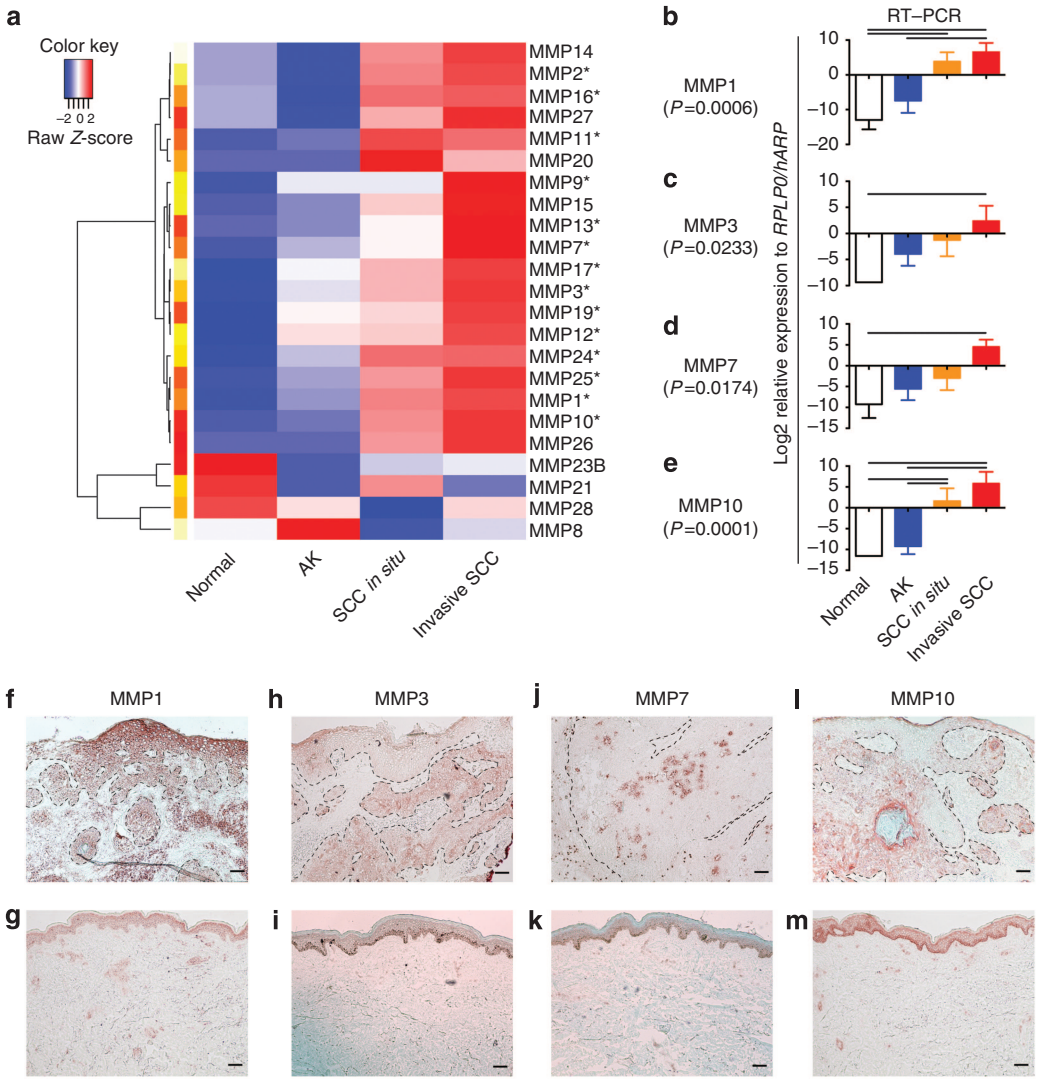

Figure 2. Mapping the regional expression differences of known matrix metallopeptidases (MMPs) was performed by reverse transcriptase–PCR (RT–PCR) and immunohistochemistry (IHC). (**a**) A heat map of mean expression of all MMPs tested across the regions is shown. AK, actinic keratosis. (**b**–**e**) RT–PCR was performed for (**b**) MMP1, (**c**) MMP3, (**d**) MMP7, and (**e**) MMP10 ($n = 5$ for each region). The *y*-axis shows relative expression level of each gene compared with the housekeeping gene *RPLP0/hARP* in Log2 (mean ± SEM). A *P*-value was estimated among four keratinocytic regions (one-way analysis of variance (ANOVA) with Tukey's correction). The resultant *P*-values are shown in parentheses. A line between two bars indicates statistical significance between two cell types. (**f**–**m**) Corresponding protein product was detected by IHC for (**f**, **g**) MMP1, (**h**, **i**) MMP3, (**j**, **k**) MMP7, and (**l**, **m**) MMP10. Upper panels indicate normal skin, and lower panels indicate squamous cell carcinoma (SCC). Dotted lines indicate borders between epidermis/tumor nests and dermis. Bar = 100 μm.

IL-24 has been shown to induce apoptosis and cell death in many solid cancers (Dash *et al.*, 2010). Its expression has been inversely correlated with lymph node metastasis or overall survival in some cancers (Ishikawa *et al.*, 2005; Patani *et al.*, 2010; Choi *et al.*, 2011). However, a phase I clinical study of intratumoral injections of Ad.IL24/mda-7 resulted in progressive disease in two cases of SCC (Cunningham *et al.*, 2005). New nodules arose around the periphery of the skin nodule of penile SCC after treatment. These results may thus suggest a putative pro-oncogenic function of IL-24 in SCC growth. Our findings may support this notion. We found that IL-24 was significantly overexpressed at the invasion nest of SCC. *IL-24* mRNA was constitutively expressed in SCC13 cells and this was further enhanced by culturing with tumor necrosis factor-α, transforming growth factor-α, and IFN-γ. The cellular source of IL-24 may, however, be more complicated within the SCC microenvironment. T helper type 2 cells and macrophages as well as melanocytes produce IL-24 (Poindexter *et al.*, 2005), and these cells reside in the SCC microenvironment. In fact, *IL-24* mRNA was also upregulated in inflammatory infiltrating cell regions surrounding tumor nests compared with normal reticular dermis (Belkin *et al.*, 2013). We thus concluded that IL-24 can be derived from multiple cell types including cancer cells within the SCC microenvironment. We have previously reported that IL-24 promoted neither proliferation nor apoptosis of A431 cells

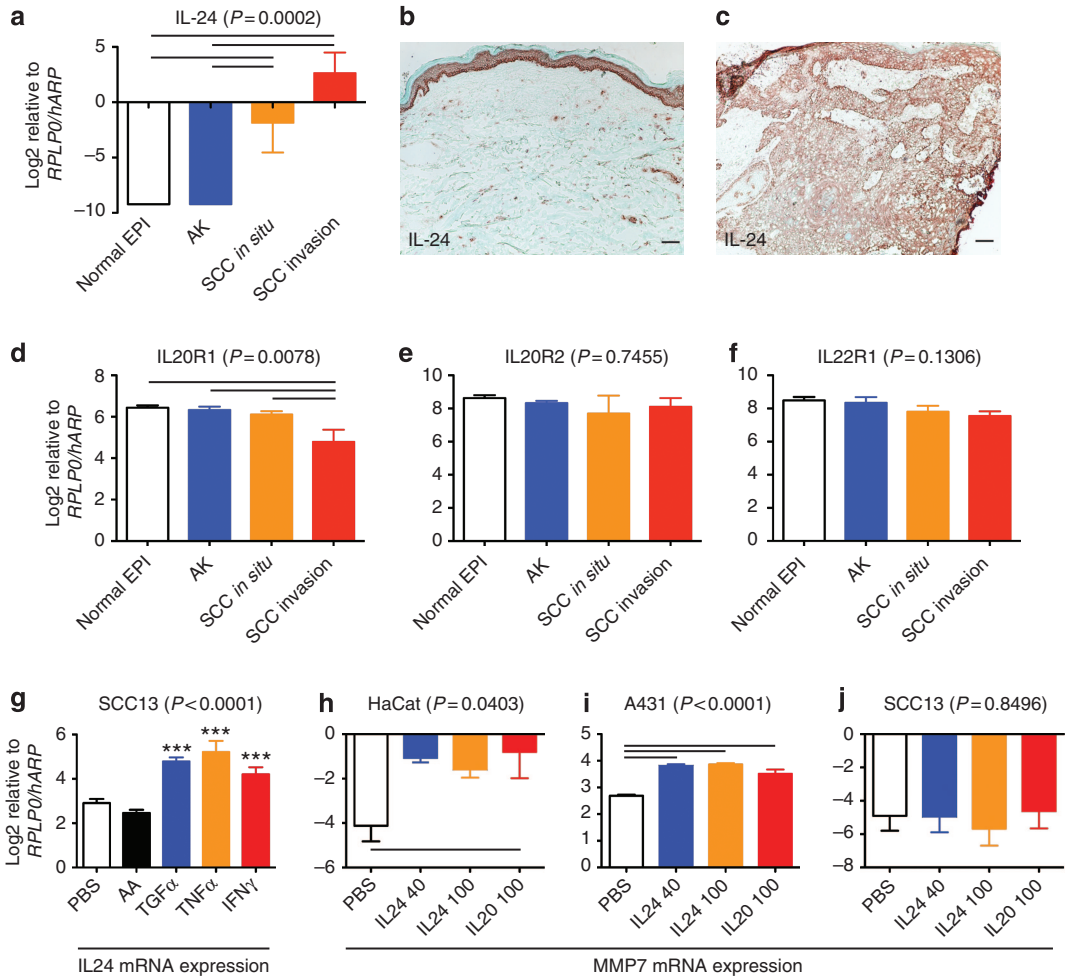

Figure 3. IL-24 induced the expression of matrix metallopeptidase 7 (MMP7) in HaCaT and A431 cells. (**a**–**c**) IL-24 expression was examined at the mRNA level (**a**) and at the protein level in (**b**) normal skin and (**c**) squamous cell carcinoma (SCC). Bar = 100 μm. (**d**–**f**) mRNA expression of (**d**) IL-20R1, (**e**) IL-20R2, and (**f**) IL-22R1 was examined. (**g**) Expression levels of IL-24 mRNA in SCC13 cells after a 12-hour treatment with phosphate-buffered saline (PBS) or indicated cytokines are shown. AA, acidic acetate; AK, actinic keratosis. (**h**–**j**) Expression of MMP7 mRNA in the three cell lines was evaluated after a 24-hour treatment with PBS, IL-24 (40 and 100 ng ml^{-1}), or IL-20 (100 ng ml^{-1}). Relative expression compared with *RPLP0*/*hARP* in Log2 is shown for all RT–PCR results (mean ± SEM). A line between two bars indicates statistical significance between the two conditions (one-way analysis of variance (ANOVA) with Tukey's correction). ***$P<0.0001$ against both PBS and AA (repeated measures ANOVA with Tukey's correction).

when cultured in a starvation condition (0.1% FBS) (Zhang *et al.*, 2013). Instead, we report herein a function of IL-24 in increasing the expression of *MMP7* in SCC cells.

The mRNA of *MMP7* was specifically upregulated in the invasion nests. The expression of MMP7 was enhanced under a hypoxic condition in colon cancer cells (Remy *et al.*, 2006). Ingenuity pathway analysis associated the genomic profile of invasive SCC with hypoxia-inducible factor-1α signaling. Thus, the microenvironment of the invasive tumor front may be prone to induce MMP7 by multiple mechanisms. We confirmed the function of MMP7 in the migration of cutaneous SCC cells. The migration of A431 cells was significantly delayed by a specific MMP7Ab in a dose-dependent manner. MMP7 has been shown to be involved in the tumor migration of various cancers through multiple pathways. One such mechanism is proposed in colon cancer cells, where MMP7 cleaves β3 chain of laminin332, resulting in enhancing migration capacity (Remy *et al.*, 2006). Laminin332 can be processed by various enzymes and its remodeling has been implicated in SCC migration (Marinkovich, 2007). All subunits of laminin332 were significantly upregulated along with SCC progression (Supplementary Table S4 online). Taken together, these results may suggest a function of MMP7 in SCC migration via accelerating laminin332 remodeling. In addition, it has been demonstrated that MMP7 shed E-cadherin and released an 80-kDa fragment of E-cadherin from A431 cells that would facilitate an epithelial-to-mesenchymal transition-like process (Shibata *et al.*, 2009). MMP7 also sheds pro-heparin-binding EGF to yield mature heparin-binding EGF (Hashimoto *et al.*, 2002). Overall, MMP7 enhances proliferation, migration, and invasion of cancer cells (Ii *et al.*, 2006). However, we acknowledge that the effect of blockade of MMP7 in 0.1% FBS-containing media was partially diminished in the 10% FBS-containing media, indicating the

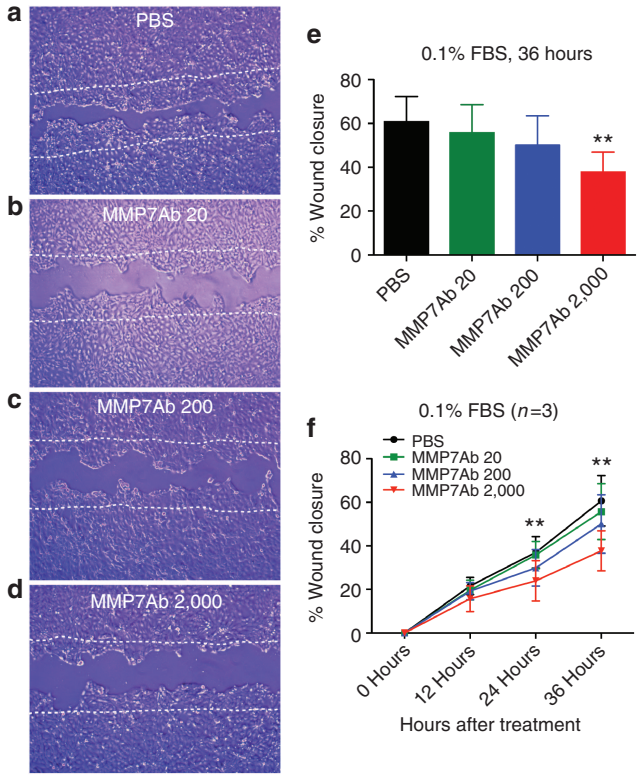

Figure 4. Blocking of matrix metallopeptidase 7 (MMP7) delayed the migration of A431 cells. Scratch was made on a culture of A431 cells at 90% confluence and cells were cultured in 0.1% fetal bovine serum (FBS)–containing media with or without indicated concentrations of anti-MMP7 antibody (MMP7Ab) for 36 hours. Cells were photographed every 12 hours. (**a**–**d**) Representative images of area after a 36-hour cultivation with (**a**) phosphate-buffered saline (PBS), (**b**) 20 ng ml^{-1} of MMP7Ab, (**c**) 200 ng ml^{-1} of MMP7Ab, and (**d**) 2,000 ng ml^{-1} of MMP7Ab. Two white dotted lines depict the initial area. (**e**) The bar graph shows mean of percent wound closure for each treatment after 36 hours. (**f**) The graph depicts the chronological change of the percent wound closure for each condition. An error bar shows SEM ($n=3$). **$P<0.01$ between MMP7Ab2,000 and PBS and MMP7Ab20 for a 24-hour treatment, and between MMP7Ab2,000 and PBS, MMP7Ab20, and MMP7Ab200 for a 36-hour treatment (repeated measures analysis of variance (ANOVA) with Tukey's correction).

contribution of other factors to the migration of A431 cells. There was a discrepancy between the expression of *IL-24* and *MMP7* in the two SCC cell lines tested. It should be noted that strong expression of *MMP7* was observed in the aggressive A431 cells. However, the less aggressive SCC13 cells and HaCaT cells, the immortalized keratinocytes, had only weak or absent expression of MMP7. This observation may support an important role of MMP7 in SCC migration. The function of IL-24 or MMP7 needs to be conclusively demonstrated using appropriate *in vivo* models. However, current mouse models of SCC do not properly recapitulate human SCC, because mouse SCCs feature follicular differentiation. Therefore, development of the animal models that accurately represent invasion of human SCC is needed.

In summary, we characterized gene expression profiling of invasion front of SCC compared with laser captured AK and SCC that have not invaded the dermis. We suggest a pro-oncogenic role of IL-24 in cutaneous SCC, unlike other cancer types, via inducing *MMP7* expression in cancer cells. Further elucidation of the mechanisms governing regulation of invasion by SCC will allow for development of targeted therapy for aggressive disease not amenable to conventional intervention.

MATERIALS AND METHODS

The detailed protocols and statistical analysis are described in the Supplementary Materials and Methods online.

Patients and samples

Institutional review board (The Rockefeller University and Weill Cornell Medical College) approval and written informed consent were obtained before enrolling patients to participate in this study. The study was performed in adherence with the Declaration of Helsinki Principles. Cutaneous SCC samples were obtained from debulking during Mohs micrographic surgery. All tumors were obtained from sun-exposed skin.

LCM

Eight SCC tissues were subjected to LCM following the manufacturer's protocol for CellCut system (Molecular Machines and Industries, Haslett, MI). Normal skin samples used in this study ($n=10$) as a reference came from our previous study (Kennedy-Crispin *et al.*, 2012).

RNA extraction and quantification

RNA extraction was performed using RNeasy Kit (QIAGEN, Valencia, CA).

RNA amplification and hybridization

Total RNA was subjected to two-cycle cDNA synthesis (Affymetrix, Santa Clara, CA), with a slight modification described previously (Mitsui *et al.*, 2012). Human Genome U133 A2.0 arrays (Affymetrix) were used. The data have been deposited to the Gene Expression Omnibus repository (GSE42677).

Cell culture

A431 cells were purchased from ATCC (Manassas, VA). SCC13 cells were kindly provided by Professor Rheinwald (Harvard Medical Center, Boston, MA). Cells were grown in appropriate media. When they reached 80% confluence, the cells were starved in empty media for 24 hours, followed by stimulation with various cytokines at indicated concentrations for 24 hours.

Scratch assay

A431 cells were grown in a 10% FBS-containing media. When the cells reached 90% confluence, a wound was made by tip of a 200 µl pipet tip and the media were replaced with either 0.1% or 10% FBS-containing media. The MMP7Ab was purchased from R&D Systems (Minneapolis, MN) and added to the culture media at the indicated concentrations. The wounds were photographed every 12 hours up to 36 hours. Measurement of wound area and calculation of percent wound closure are described in the Supplementary Materials and Methods online.

Quantitative RT–PCR

Preamplification quantitative RT–PCR technique was used for measuring mRNA expression values in total RNA extracted from

microdissected samples according to the company's instructions (Applied Biosystems, Foster City, CA). Regular TaqMan RT–PCR method was used to detect the signals in total RNA extracted from cultured cells. All data were normalized to RPLP0/hARP. Primers and probes used in this experiment are listed in Supplementary Table S7 online.

Immunohistochemistry

Frozen skin sections were prepared and standard procedures were used. Antibodies used in this experiment are listed in Supplementary Table S8 online.

Statistical analysis

Microarray data were analyzed using R/Bioconductor packages (www.r-project.org). The Harshlight package (Suárez-Fariñas *et al.*, 2005) was used to scan Affymetrix chips for spatial artifacts. Expression values were obtained using gcrma algorithm. Expression values were linearly modeled in the *limma* package framework. For the comparison of interest, the moderated *t*-test was used to assess differential expression. *P*-values for each comparison were adjusted for multiple hypotheses using the Benjamini–Hochberg approach. Genes with false discovery rate of <0.05 and fold change of >3.0 were considered as differentially expressed.

Statistical analyses of all RT–PCR data were performed using Graphpad Prism ver.5 (GraphPad Software, La Jolla, CA). A $P<0.05$ was considered as statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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